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Note

Isocratic high-performance liquid chromatography of bile pigments

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The first application of high-performance liquid chromatography (HPLC) to pyrrolic pigments of animal bile appeared in a preliminary report from this laboratory [1] in which isocratic separation of azodipyrroles of bilirubin and its conjugates from dog bile was described. Gradient elution has subsequently been employed [2] to separate these compounds, requiring longer analysis times, more elaborate equipment and re-equilibration of columns between samples.

Separation of bile pigments as underivatized tetrapyrroles has recently been described by several investigators [3—5]. For many purposes, however, the use of azodipyrrole derivatives remains desirable because of greatly enhanced stability and less complex chromatograms.

At the time our original procedure [1] was published, the identities of some of the HPLC peaks were uncertain. We have now established the identities of all of the components resolved from dog bile and have modified the procedure to improve the resolution and extend its application to biliverdin isomers and non-α isomers of bilirubin.

EXPERIMENTAL

Chemicals

Reagent grade solvents were obtained from Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.) and were filtered through 0.45-μm Fluoropore filters (Millipore) before use. Human serum albumin (fraction V), hemin (type 1), bacterial β-glucuronidase (type I), β-glucosidase (almond), uridine-5′-diphosphoglucuronic acid (ammonium salt), uridine-5′-diphosphoglucose (sodium salt), uridine-5′-diphosphoxylose, D-saccharic acid-1,4-lactone and D-gluconic acid lactone (grade IX) were ob-

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tained from Sigma (St. Louis, MO, U.S.A.). Biliverdin IXα (dihydrochloride) and bilirubin IXα were obtained from Porphyrin Products (Logan, UT, U.S.A.). *p*-Iodoaniline (Fisher Scientific) was recrystallized from hexane and stored at −80°C until used.

Apparatus

All experiments employed ALC 200 series liquid chromatographs (Waters Assoc., Milford, MA, U.S.A.) equipped with Model U6K injectors and Model 440 absorbance detectors operating at fixed wavelengths of 546 nm or 365 nm. Columns (150 × 4.6 mm) were obtained from various sources prepacked with LiChrosorb 5 RP-8 (5 μm). Differences among columns from various suppliers were negligible.

Preparation of samples for chromatography

Bile was collected from the gallbladders of anesthetized dogs via hypodermic needle. The bile pigments were stabilized by addition of one volume of citrate—phosphate buffer, pH 4 [6] and storage at −80°C protected from light. Diazo coupling of the bile pigments with *p*-iodoaniline was performed as described by Heirwegh et al. [7]. Small aliquots of the organic extracts were dried at 30°C by a gentle stream of nitrogen. Dried extracts were either used immediately or stored, protected from light, at −80°C. The samples were redissolved in methanol for thin-layer chromatography (TLC) or in methanol—ethyl acetate (1 : 1) for HPLC.

Thin-layer chromatography

Established TLC procedures were employed to obtain pure materials for use in identifying the components separated by HPLC. All experiments were performed in the dark at ambient temperature using precoated 20 × 20 cm silica gel plates (DC-Kieselgel, E. Merck, Darmstadt, G.F.R.).

Azopigments of bilirubin, bilirubin glucuronide, bilirubin glucoside and bilirubin xyloside were separated by development with either chloroform—methanol—water (65 : 25 : 3) or chloroform—methanol (17 : 3) [8]. The major components resolved by this method were designated by the authors as α_0 (azodipyrrole), α_2 (azodipyrrole xyloside), α_3 (azodipyrrole glucoside) and δ (azodipyrrole glucuronide). We have chosen to use the same designations.

Endovinyl and exovinyl azodipyrroles of unconjugated bilirubin were resolved by repeated development with chloroform—ethyl acetate (1 : 1) [9].

High-performance liquid chromatography

Separations were performed at ambient temperature with a flow-rate of 1.0 ml/min. Column inlet pressures varied from 3.5 to 10 MPa.

Mobile phases contained acetonitrile, ethyl acetate, methanol, water and tetrabutylammonium counter-ion (added as tetrabutylammonium hydroxide, 40% aqueous solution). The pH and water content were varied according to the application, and the counter-ion concentration was maintained at 10 m*M*. To prepare the mobile phase 30 ml acetonitrile, 33 ml ethyl acetate,

45 ml methanol and 50 ml water are mixed. The appropriate quantity of counter-ion is then added and the pH adjusted with 4.4 *N* phosphoric acid (mobile phases A and B) or 2.2 *N* phosphoric acid—acetic acid mixture (mobile phases C and D). Finally, the mobile phase is made up to the final volume with water and the pH checked and readjusted if necessary. The mobile phases utilized in this investigation differed as follows: Mobile phase A: pH 6.1; counter-ion 1.3 ml; final volume 200 ml. Mobile phase B: pH 6.1; counter-ion 1.0 ml; final volume 155 ml. Mobile phase C: pH 4.25; counter-ion 1.3 ml; final volume 200 ml. Mobile phase D: pH 4.0; counter-ion 1.6 ml; final volume 240 ml.

RESULTS AND DISCUSSION

In the original procedure [1] the azodipyrroles of unconjugated bilirubin eluted immediately behind the azodipyrrole glucuronides and were difficult to consistently separate from the glucuronides and later-eluting components. By lowering the pH to 6.1 we were able to considerably increase the retention of the unconjugated pigments eliminating that problem. We also changed from a C-18 to a C-8 column which reduces the analysis time.

Separation and identification of azopigments of dog bile

Fig. 1a shows typical results of HPLC of extracted *p*-iodoaniline azopigments of dog bile. Reaction of bilirubin with diazonium salts results in cleavage at the central methylene bridge yielding two isomeric azodipyrroles which are resolved by our procedure. Peaks 7 and 8 have retention times identical to azodipyrroles of commercial bilirubin. Peaks 1—6 are quantitatively converted to azodipyrrole methyl esters upon reaction with methanolic sodium hydroxide [10] indicating that these components are ester conjugates of bilirubin. To aid in establishing the specific identities of peaks 1—6 their retention times were compared to those of azopigments isolated by the TLC procedure of Heirwegh et al. [8]. Each TLC azopigment was resolved into two peaks of approximately equal areas whose retention times corresponded to pairs of peaks in Fig. 1a as indicated. The identities of peaks 1—6 were confirmed by comparison with azodipyrroles of bilirubin conjugates synthesized enzymatically using specific substrates (Figs. 1b—d). A digitonin-activated rat liver crude homogenate prepared as described by others [11, 12] was used as a source of bilirubin UDP-glycosyl transferases. The homogenates were incubated with either UDP-glucuronic acid (UDPGA), UDP-glucose (UDPG) or UDP-xylose (UDPX). Aliquots of the reaction mixtures were derivatized and prepared for HPLC in the same manner as bile specimens. When UDPGA was the substrate (Fig. 1b) the products of the reaction had the same retention times as azopigments 1 and 2 of dog bile. With UDPG as the substrate (Fig. 1c) the reaction products had the same retention times as dog bile azopigments 3 and 4. With UDPX as the substrate (Fig. 1d) the products had the same retention times as dog bile azopigments 5 and 6. The identities of peaks 1—4 were further confirmed by enzymatic hydrolysis. Incubation of extracted azopigments with bacterial β-glucuronidase resulted in the disappearance of peaks 1 and 2 which was inhibited by saccharic acid-1,4-lactone.

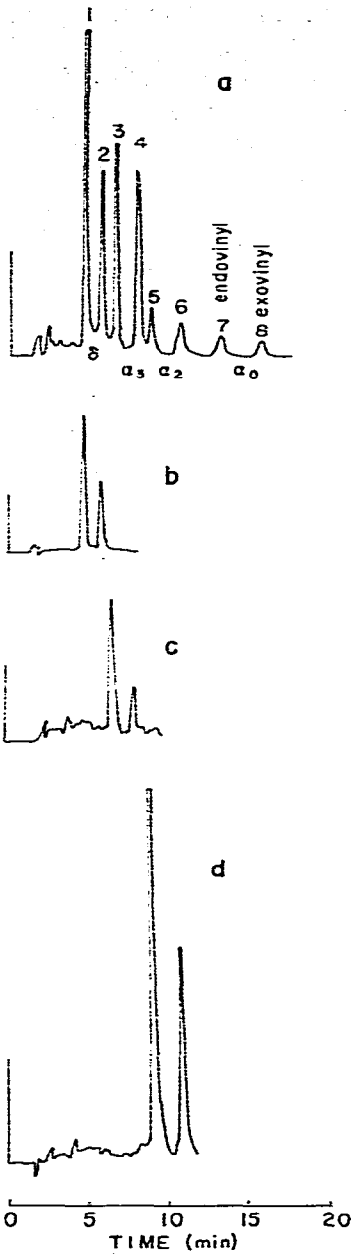

Fig. 1. (a) HPLC of *p*-iodophenyl azodipyrroles of dog bile with mobile phase A. Peaks: (1) endovinyl azodipyrrole glucuronide; (2) exovinyl azodipyrrole glucuronide; (3) endovinyl azodipyrrole glucoside; (4) exovinyl azodipyrrole glucoside; (5) endovinyl azodipyrrole xyloside; (6) exovinyl azodipyrrole xyloside; (7) endovinyl azodipyrrole; (8) exovinyl azodipyrrole. (b) HPLC of azodipyrroles of enzymatically synthesized bilirubin glucuronide (mobile phase A). (c) HPLC of azodipyrroles of enzymatically synthesized bilirubin glucoside (mobile phase A). (d) HPLC of azodipyrroles of enzymatically synthesized bilirubin xyloside (mobile phase A).

Incubation with almond β-glucosidase resulted in the disappearance of peaks 3 and 4 which was inhibited by gluconic acid lactone.

To establish the order of elution of isomeric azodipyrroles we separated unconjugated endovinyl and exovinyl isomers by TLC with multiple development and subjected the isolated isomers to HPLC. The endovinyl isomer was the first to elute, corresponding to azopigment 7 of dog bile (Fig. 1a). The exovinyl isomer had the same retention time as azopigment 8 of dog bile. To determine whether the endovinyl and exovinyl conjugated azodipyrroles eluted in the same order as the unconjugated pigments, we individually injected into the HPLC system the TLC-separated azopigments α_2, α_3, and δ, collecting each azodipyrrole immediately as it emerged from the flow cell. Reinjection confirmed that the isomers had been isolated in pure form. The isolated azodipyrroles were enzymatically deconjugated and rechromatographed by HPLC. In each case the endovinyl/exovinyl order was the same for the conjugated and unconjugated pigments.

Separation of biliverdin isomers

Biliverdin dimethyl esters were synthesized as described by Bonnett and McDonagh [13] and were separated by TLC [14]. The purified biliverdin dimethyl esters were converted to free acids by treatment with sodium hydroxide in methanol [14]. The resulting biliverdin isomers were extracted into chloroform at pH 2.7, dried under nitrogen and subjected to HPLC using mobile phase D. Retention times on HPLC were determined for the individual isomers. A high-performance liquid chromatogram of a mixture of the four biliverdin isomers is shown in Fig. 2.

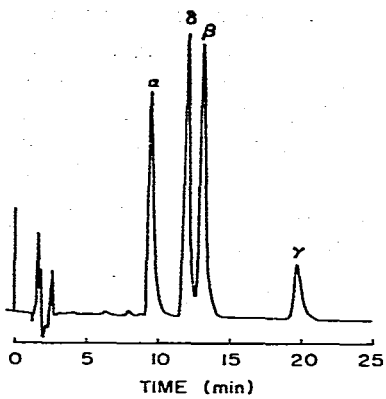

Fig. 2. HPLC of biliverdin isomers (mobile phase D).

Separation of azodipyrroles of non-α isomers of bilirubin

In order to investigate the behavior of azopigments of bilirubin IXβ, IXγ, and IXδ in our HPLC system biliverdin isomers were reduced with sodium borohydride [14] to bilirubin isomers. Azodipyrrole derivatives were prepared in the usual manner.

Fig. 3 shows the separation of azodipyrroles of dog bile mixed with biliverdin IXα and the azodipyrroles of bilirubin IXβ, IXγ, and IXδ. Bilirubin

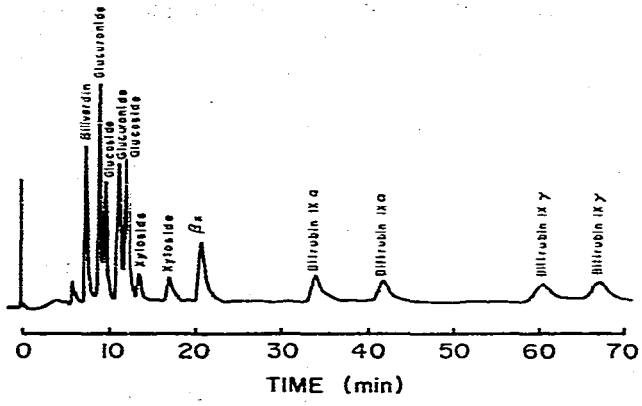

Fig. 3. HPLC of azodipyrroles of dog bile mixed with biliverdin IXα and azodipyrroles of bilirubin IXβ, IXγ, and IXδ (mobile phase C).

IXβ and IXδ produce an azodipyrrole in common which contains two propionic acid side chains and was designated βx by Heirwegh et al. [8]. This pigment is not reliably resolved from the azopigments of bilirubin glucuronide in our standard system (mobile phase A), but by lowering the pH to 4.25 (mobile phase C) azopigment βx can be made to elute just behind the azopigments of bilirubin xyloside. This modification also resulted in slower migration of the azodipyrrole glucuronides so that the exovinyl azodipyrrole glucuronide eluted between the two azodipyrrole glucosides. The azodipyrroles of bilirubin IXγ elute just behind those of unconjugated bilirubin IXα in either mobile phase A or mobile phase C. In addition to azodipyrrole βx, bilirubin IXβ yields an azodipyrrole designated α_F and bilirubin IXδ yields an azodipyrrole designated α_F' [14]. These azopigments contain no polar side chains and, as a result, are very strongly retarded in mobile phase A or C. Mobile phase B, containing less water, will elute azopigments α_F and α_F' making it possible to establish the relative amounts of bilirubin IXβ and IXδ in samples containing azopigment βx. The azodipyrroles of all four bilirubin isomers are separated from each other with mobile phase B (Fig. 4) but this system will not resolve conjugated azodipyrroles from each other or from azopigment βx.

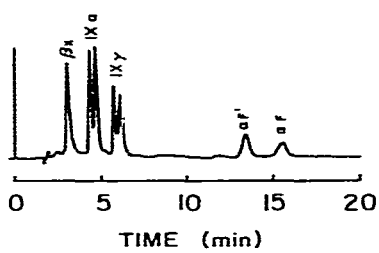

Fig. 4. HPLC of azodipyrroles of bilirubin isomers (mobile phase B).

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